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1 **Title:** Horizontal transfer of the *bla*_{NDM-1} gene to *Pseudomonas aeruginosa* and *Acinetobacter*
2 *baumannii* within a biofilm

3 **Running title:** Transfer of *bla*_{NDM-1} into *P. aeruginosa* and *A. baumannii*

4

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19

20 **ABSTRACT**

21 Horizontal gene transfer has contributed to the global spread of the *bla*_{NDM-1} gene. Studies
22 have demonstrated plasmid transfer of *bla*_{NDM-1} into various Gram-negative bacterial species, but
23 attempts to demonstrate transfer of *bla*_{NDM-1} plasmids into *Pseudomonas aeruginosa* and
24 *Acinetobacter baumannii* have either been unsuccessful or only observed with a donor of the
25 same genus. There is evidence that plasmid transfer frequency may increase when conjugation
26 occurs within a biofilm versus between planktonic cells. To determine whether *bla*_{NDM-1} gene
27 transfer to *P. aeruginosa* or *A. baumannii* could be facilitated in a biofilm environment, one *E.*
28 *coli* and two *Klebsiella pneumoniae* strains carrying NDM-1-encoding plasmids of different
29 incompatibility types were mated with an *E. coli* J53 strain to produce *E. coli* J53- *bla*_{NDM-1}
30 transconjugant plasmid donors. Dual-species biofilms were then created using the *E. coli* J53
31 transconjugants and a *P. aeruginosa* or *A. baumannii* recipient strain and incubated for 24 or 72
32 hours. Transfer of an NDM-encoding plasmid from one *E. coli* J53- *bla*_{NDM-1} transconjugant into
33 *P. aeruginosa* was successful in a 72-hour biofilm, and transfer of NDM-encoding plasmids
34 from two *E. coli* J53-*bla*_{NDM-1} transconjugants to *A. baumannii* was successful in 24-hour
35 biofilms.

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Deleted: Biofilm transfer of NDM-encoding plasmids to these bacterial species has serious implications for community and healthcare environments.

52 INTRODUCTION

53 ~~Spread of the~~ *bla*_{NDM-1} carbapenemase gene is ~~a global~~ public health concern
54 (Kumarasamy *et al.*, 2010). The gene ~~is typically mobile, commonly~~ carried on plasmids of
55 diverse sizes and incompatibility types that are ~~capable of inter-species, inter-genus, and inter-~~
56 family transfer. (Carattoli, 2013). ~~Successful dissemination of the~~ *bla*_{NDM-1} gene ~~is more~~
57 ~~commonly attributed to conjugational transfer of NDM-1-encoding plasmids to other Gram-~~
58 ~~negative bacteria, rather~~ than clonal spread (Carattoli, 2013, Johnson & Woodford, 2013).
59 The *bla*_{NDM-1} gene ~~is typically found in~~ *Enterobacteriaceae* species, ~~but~~ has ~~also~~ been
60 detected in a variety of ~~non-fermenting Gram-negative bacteria~~, such as *Aeromonas caviae*,
61 *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*, ~~and several~~ *Pseudomonas* species,
62 ~~including~~ *Pseudomonas aeruginosa* (Walsh *et al.*, 2011, Zhang *et al.*, 2013). ~~Dissemination of~~
63 ~~the~~ *bla*_{NDM-1} gene in *P. aeruginosa* and *A. baumannii* isolates has now been reported globally in
64 both clinical and environmental samples, and treatment options have become significantly
65 limited (Chen *et al.*, 2011, Chaudhary & Payasi, 2013). ~~Many of these isolates likely acquired~~
66 ~~the~~ *bla*_{NDM-1} gene via intra- or inter-genus conjugational transfer of NDM-1-encoding plasmids;
67 ~~however, successful inter-family~~ transfer of the *bla*_{NDM-1} ~~harboring plasmids to~~ *P. aeruginosa* or
68 *A. baumannii* has not yet been demonstrated in the laboratory (Potron *et al.*, 2011, Janvier *et al.*,
69 2013, Huang *et al.*, 2015).

70 ~~Conjugation experiments using Gram-positive donors and recipients typically use surface~~
71 ~~mating approaches such as filter mating or biofilm formation~~ (Roberts *et al.*, 2001, Savage *et al.*,
72 2013). ~~However, because pili can assist in gene transfer in Gram-negative bacteria, broth mating~~
73 ~~has been a commonly used method for conjugational transfer of NDM-encoding plasmids in~~
74 ~~Gram-negative species~~ (Potron *et al.*, 2011, Sowmiya *et al.*, 2012, Rahman *et al.*, 2014).

Deleted: The rapid global increase in hospital- and community-acquired carbapenem-resistant infections is an urgent public health threat, and very few treatment options remain (Centers for Disease Control and Prevention, 2013). Carbapenem resistance mediated by carbapenemase enzymes is of particular concern, as the encoding genes are often found on mobile genetic elements that can be transferred horizontally to other bacterial species (Johnson & Woodford, 2013). ¶

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104 Bacterial species such as *P. aeruginosa* and *A. baumannii*, can frequently be recovered from
105 hospital or natural environments (Blanc *et al.*, 2007, Walsh *et al.*, 2011, Nutman *et al.*, 2016),
106 and are often found in a sessile or biofilm state (Donlan, 2002, Gurung *et al.*, 2013). Studies have
107 demonstrated that horizontal gene transfer can occur at higher frequencies in biofilms versus
108 planktonic cells in Gram-negative bacterial species (Madsen *et al.*, 2012). The high density and
109 close spatial proximity of the cells create an ideal environment for interspecies transfer of genetic
110 information (Donlan, 2002, Madsen *et al.*, 2012). *P. aeruginosa* and *A. baumannii* are commonly
111 associated with biofilm formation (Donlan, 2002, Qi *et al.*, 2016), which could potentially
112 facilitate the transfer of *bla*_{NDM-1} to these bacteria. The objective of this study was to determine
113 whether plasmid-borne *bla*_{NDM-1} genes originating in *Enterobacteriaceae* species could be
114 transferred from an *E. coli* J53-*bla*_{NDM-1} transconjugant to *P. aeruginosa* or *A. baumannii* in a
115 biofilm environment.

116 **MATERIALS AND METHODS**

117 **Donor and recipient organisms**

118 Four NDM-1-producing *Enterobacteriaceae* strains with plasmids of different
119 incompatibility types carrying the *bla*_{NDM-1} gene were used as the original plasmid donors: a *K.*
120 *pneumoniae* donor (EKP) carrying the *bla*_{NDM-1} gene on a 100 kb plasmid (pEKP) belonging to
121 the FII, L/M, or N2 incompatibility group; an *E. coli* donor (EEC) carrying the *bla*_{NDM-1} gene on
122 a 150 kb plasmid (pEEC) belonging to the FII incompatibility group; a *K. pneumoniae* donor
123 (CO-NDM) carrying the *bla*_{NDM-1} gene on a 130 kb plasmid (pCO-NDM) of unknown
124 incompatibility type; and a *K. pneumoniae* donor (ATCC BAA-2146) carrying the *bla*_{NDM-1} gene
125 on a 140 kb plasmid (pNDM-US) belonging to the A/C incompatibility group (Hudson *et al.*,
126 2014). EKP and EEC were recovered from environmental samples in Southeast Asia, and the

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132 CO-NDM and ATCC strains were isolated from clinical samples. Azide-resistant *E. coli* J53 was
133 used as a recipient for mating experiments with the original plasmid donor strains. Subsequent
134 mating-out assays were performed using the *E. coli* J53-*bla*_{NDM-1} transconjugants as donors and
135 rifampin-resistant *P. aeruginosa* and *A. baumannii* as recipients. *E. coli* J53-*bla*_{NDM-1}
136 transconjugants were used as donors to allow for comparison of transfer frequencies between
137 each of the NDM-encoding plasmid types, as previously described (Potron *et al.*, 2011).

138 **Broth conjugations into azide-resistant *E. coli* J53**

139 Log phase Luria-Bertani (LB) broth cultures of each of the *bla*_{NDM-1} donors and the *E.*
140 *coli* J53 recipient were combined in a 10:1 donor-to-recipient ratio in fresh LB. Mating-out
141 assays were performed as described by Walsh *et al.* (Walsh *et al.*, 2011) using LB rather than
142 nutrient broth. Conjugation mixtures were incubated overnight at 30 and 37 degrees C, then
143 serially diluted and plated on LB agar containing 0.5 µg/mL meropenem and 100 µg/mL sodium
144 azide.

145 Transfer of *bla*_{NDM-1} into *E. coli* J53 was confirmed by PCR with a previously described
146 primer set (Poirel *et al.*, 2011) and on CHROMagar Orientation™ (DRG International,
147 Springfield, New Jersey) containing 0.5 µg/mL meropenem. Putative NDM-1-positive *E. coli*
148 J53 transconjugants from the EEC donor were differentiated from the parent EEC strain by PCR
149 detection of the *yja-A* gene found in *E. coli* J53 but absent in the EEC strain (Clermont *et al.*,
150 2000). Three of the *bla*_{NDM-1} donors, EKP, EEC, and ATCC-BAA-2146 produced *E. coli* J53
151 transconjugants, designated *E. coli* TcEKP, *E. coli* TcEEC, and *E. coli* TcNDM-US,
152 respectively.

153 **Biofilm conjugations into rifampin-resistant *P. aeruginosa* and *A. baumannii***

Deleted: Details on the four NDM-1-producing *Enterobacteriaceae* strains used as *bla*_{NDM-1} donors in the first phase of the conjugation assays are given in Table 1.

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163 Biofilm mating-out assays were performed using NDM-1-positive *E. coli* J53 transconjugants
164 TcEKP, TcEEC, and TcNDM-US as *bla*_{NDM-1} plasmid donors and *P. aeruginosa* and *A.*
165 *baumannii* as recipients. Using optical density measurements, log-phase donor and recipient LB
166 broth cultures were combined in a 1:4 donor-to-recipient ratio, with approximately 2.5 x 10⁷ cells
167 from an *E. coli* J53 transconjugant donor and 1.0 x 10⁸ cells from the *P. aeruginosa* or *A.*
168 *baumannii* recipient in 1 mL LB.

169 The 1 mL dual-species conjugation mixtures were placed in a 48-well plastic plate and
170 incubated for either 24 or 72 hours, allowing the culture to form a biofilm on the sides of the
171 wells. Conjugation mixtures were incubated at 30 degrees for the *P. aeruginosa* conjugations and
172 37 degrees for the *A. baumannii* conjugations. LB was exchanged every 24 hours to maintain
173 nutrient levels. At the end of the incubation period the broth was again exchanged, and biofilms
174 were scraped from the well sides using a sterile metal scraper. The LB containing the biofilm
175 scrapings was pulse-vortexed to break apart the cells, and serial dilutions of the biofilm
176 suspension were plated on tryptic soy agar containing 75 µg/mL ticarcillin and 50 or 100 µg/mL
177 rifampin for *A. baumannii* or *P. aeruginosa*, respectively. Plates were incubated at 37 degrees C
178 for 48 hours.

179 Colonies from ticarcillin-rifampin selection plates were subcultured to CHROMagar™
180 with 0.5 µg/mL meropenem. *P. aeruginosa* or *A. baumannii* colony lysates from the
181 CHROMagar™ plates were tested for the *bla*_{NDM-1} gene by PCR, as described above.

182 **Minimum inhibitory concentrations**

183 Minimum inhibitory concentrations (MICs) of all *bla*_{NDM-1} donors, recipients, and
184 transconjugants were determined by meropenem Etest® (bioMérieux Clinical Diagnostics,
185 Marcy l'Etoile, France).

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- Moved down [1]: *P. aeruginosa* or *A. baumannii* recipient
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- Deleted: Cultures of each NDM-1-positive *E. coli* J53 transconjugant and the *P. aeruginosa* or *A. baumannii* recipient were combined in a 1:4 donor-to-recipient ratio in LB, as previously described (Potron *et al.*, 2011), with broth mating assays otherwise performed as detailed above.
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- Deleted: When *bla*_{NDM-1} biofilm transfer to *P. aeruginosa* or *A. baumannii* was successful, NDM-1-positive *E. coli* J53 donors were used to create a "layered" biofilm to confirm biofilm transfer. Single species 24-hour *P. aeruginosa* and *A. baumannii* biofilms were formed and then rinsed with LB, followed by addition of an NDM-1-positive *E. coli* J53 cultures to each biofilm well. Following a 48-hour incubation, with daily LB exchange, biofilms were then harvested and plated as described above.¶

217 **Plasmid analysis**

218 Genetic location (plasmid or chromosome) of the *bla*_{NDM-1} gene, after conjugational
219 transfer was determined by a combination of Pulsed Field Gel Electrophoresis (PFGE) of S1
220 digested macro DNA of the various plasmid donor strains and transconjugants followed by
221 detection using ³²P labelled *bla*_{NDM-1} and *bla*_{CMY-2} probes using methods described by Patzer et al
222 2009 (Patzer *et al.*, 2009). Probes were prepared by PCR using primers pairs: NDMF/R
223 TGGCTTTTGAAACTGTGCGCACC, CTGTACATCGAAATCGCGCGA; CMY2F/R
224 AAATCGTTATGCTGCGCTCT, GACACGGACAGGGTTAGGAT, respectively.

225 **RESULTS**

226 **Biofilm conjugations into *P. aeruginosa* and *A. baumannii***

227 Transfer frequencies of the *bla*_{NDM-1} plasmids from the *E. coli* J53-*bla*_{NDM-1}
228 transconjugants to *P. aeruginosa* or *A. baumannii* are presented in Table 1. Transfer of the
229 *bla*_{NDM-1} plasmid pNDM-US to *P. aeruginosa* from TcNDM-US, was successful in a 72-hour
230 biofilm, but was not detected in a 24-hour biofilm. Transfer of the *bla*_{NDM-1} plasmids pNDM-US
231 and pEKP from the TcNDM-US and TcEKP to *A. baumannii* was successful in a 24-hour
232 biofilm, but was not detected in a 72-hour biofilm.

233 **Minimum inhibitory concentrations**

234 All parental *bla*_{NDM-1} donor strains had meropenem MICs greater than 32 µg/mL. All
235 transconjugants had meropenem MICs of 24 µg/mL or greater.

236 **Plasmid analysis**

237 S1 PFGE and *bla*_{NDM-1} ³²P labeled probe showed that the ATCC BAA-2146 *bla*_{NDM-1}
238 donor harbored a NDM-encoding plasmid of the approximately 140 kb. An NDM-encoding
239 plasmid of the same size was found in the *P. aeruginosa-bla*_{NDM-1} transconjugant, *P. aeruginosa*

Deleted:	and <i>bla</i> _{CMY-2} resistance
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Deleted:	Broth conjugations into azide-resistant <i>E. coli</i> J53¶ Figure 1 shows the overall sequence of the mating-out assays and results. Transfer rates of NDM-encoding plasmids from the ATCC BAA-2146 <i>K. pneumoniae</i> , EKP, and EEC donor strains to <i>E. coli</i> J53 are listed in Table 1. ¶ Broth and
Deleted:	Conjugations
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286 TcATCC, and in the *A. baumannii*-*bla*_{NDM-1} transconjugant, *A. baumannii* TcATCC. Analysis of
 287 the EKP *bla*_{NDM-1} donor by S1 PFGE and *bla*_{NDM-1} ³²P labeled probe showed that the *bla*_{NDM-1}
 288 gene was located on a 100kb plasmid and a *bla*_{NDM-1} plasmid of the same size was found in the
 289 *A. baumannii*-*bla*_{NDM-1} transconjugant, *A. baumannii* TcEKP. S1 PFGE analysis did not show
 290 insertion of the *bla*_{NDM-1} gene into the chromosome of either *P. aeruginosa* or *A. baumannii*.

291 DISCUSSION

292 We successfully transferred plasmids carrying the *bla*_{NDM-1} gene from *Klebsiella* and *E.*
 293 *coli* donor strains into *E. coli* J53, and subsequently from the *E. coli* J53-*bla*_{NDM-1}
 294 transconjugants into *P. aeruginosa* and *A. baumannii*. Plasmid analyses by S1 PFGE and *bla*_{NDM-1}
 295 ³²P labeled probe indicated that the *bla*_{NDM-1} gene remained plasmid-located in the *P.*
 296 *aeruginosa* and *A. baumannii* *bla*_{NDM-1} transconjugants in our study. To our knowledge, plasmid
 297 transfer of the *bla*_{NDM-1} gene into *P. aeruginosa* has not been observed experimentally, and
 298 plasmid transfer of *bla*_{NDM-1} into *A. baumannii* has previously only been demonstrated by
 299 electroporation (Potron *et al.*, 2011), or using agar surface mating techniques and an
 300 *Acinetobacter* donor (Huang *et al.*, 2015). Agar and filter surface mating methods, like biofilm,
 301 provide a stable, spatially-structured environment for gene transfer; however, sites of nutrient
 302 uptake and gas exchange differ, and less of the protective extracellular polymeric substance is
 303 produced in agar colonies compared to biofilms (Davey & O'Toole, 2000, Stalder & Top, 2016).
 304 Additionally, the plasmids donors in our assays were *Enterobacteriaceae* species and inter-
 305 family transfer via *E. coli* J53-*bla*_{NDM-1} transconjugants to *P. aeruginosa* and *A. baumannii* has
 306 not previously been demonstrated with other NDM-1-encoding plasmids.
 307 NDM-1 mating assays described in the peer-reviewed literature frequently use broth
 308 mating techniques (Potron *et al.*, 2011, Zhang *et al.*, 2013, Ou *et al.*, 2014). We did not observe

Deleted: and ethidium bromide staining (Figure 2 (b)). Probing of the gel with a *blac*_{MY-2} ³²P labeled probe (Figure 2 (a)) indicated that the *blac*_{MY-2} gene was associated with a 140kb plasmid in the *K. pneumoniae* ATCC donor only and probing of a replicate gel (Figure 2 (c)) indicated that the same 140kb plasmid was associated with the *bla*_{NDM-1} gene. Probed gels also indicated that transfer of the 140kb plasmid to *P. aeruginosa* and *A. baumannii* occurred as a result of mating the recipient strains with the *E. coli* J53-*bla*_{NDM-1} ATCC intermediate donor (Figure 2 (a-c), Figure 3). Interestingly, in one of the *P. aeruginosa* transconjugants the *blac*_{MY-2} and *bla*_{NDM-1} positive plasmid was slightly smaller (130kb). The probed gel indicated that the *blac*_{MY-2} and *bla*_{NDM-1} positive plasmid in *P. aeruginosa* was found in various multimeric forms.

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358 plasmid transfer of *bla*_{NDM-1} to *P. aeruginosa* in planktonic broth cultures, similar to other studies
359 (Potron *et al.*, 2011, Janvier *et al.*, 2013), and only pEKP transferred in broth to *A. baumannii*
360 (data not shown). Transfer of pEEC to either recipient was not detected. It should be noted that
361 transfer of pEEC, or higher transfer rates of pEKP and pNDM-US donors, might have been
362 observed if mating with the *P. aeruginosa* and *A. baumannii* recipients had been performed using
363 the original parent *bla*_{NDM-1} donor, rather than via an *E. coli* J53 transconjugant. Mating was
364 performed using an *E. coli* J53- *bla*_{NDM-1} transconjugant to enable comparison of transfer
365 frequency of the different NDM-1 plasmid types and comparison with previous attempts to
366 transfer *bla*_{NDM-1} plasmids into *P. aeruginosa* and *A. baumannii* (Potron *et al.*, 2011). Prior
367 studies have been unable to detect transfer of NDM-encoding plasmids to *P. aeruginosa* or *A.*
368 *baumannii* from *Enterobacteriaceae* donors by conjugation under broth conditions. This work
369 demonstrates that conjugative inter-family transfer of these plasmids can be successful when
370 mating occurs in a biofilm environment.

371 ↓

372 **Funding Information**

373 This work was supported by the Health Studies Fund of the Department of Family and
374 Preventive Medicine of the University of Utah.

375 **Conflict of Interest**

376 The authors have no conflicts of interest to declare.

377 **Acknowledgements**

378 We wish to thank the Utah Public Health Laboratory for their assistance and the use of their
379 facility for part of the PCR analyses of these isolates. We are also grateful to the Colorado
380 Department of Public Health & Environment Laboratory Services Division for providing the

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Moved up [2]: While studies of clinical NDM-1-producing *P. aeruginosa* isolates have found the *bla*_{NDM-1} gene located on the chromosome in *P. aeruginosa* (Janvier *et al.*, 2013, Jovicic *et al.*, 2014), the gene remained plasmid-located in both the *P. aeruginosa* and *A. baumannii* transconjugants in our study.

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Deleted: The plasmid was of identical size (140 kb) to the ATCC donor in one *P. aeruginosa* transconjugant but a second transconjugant was slightly smaller (approx. 130kb). This is likely due to a deletion event, which appears to be a common event during plasmid transfer of NDM-encoding plasmids (Kumarasamy *et al.*, 2010). Interestingly, in both *P. aeruginosa* transconjugants the NDM-encoding plasmids were found in multiple forms which are most likely multimeric forms of the same plasmid, which was also partially visible in the donor strain (Figure 2). Multiple copies of the *bla*_{NDM-1} gene on the *P. aeruginosa* chromosome have been reported previously (Jovicic *et al.*, 2014), and similar multimeric plasmid forms have also been seen in NDM-encoding plasmids in *Acinetobacter* species (Jones *et al.*, 2014).¶
In the U.S., healthcare-acquired multidrug-resistant *Acinetobacter* and *P. aeruginosa* are responsible for an estimated 7300 and 6700 infections each year, respectively (Centers for Disease Control and Prevention, 2013). Biofilm-forming *P. aeruginosa* and *A. baumannii* have been implicated in healthcare facility outbreaks traced to environmental sources (Hota *et al.*, 2009, Doidge *et al.*, ...

459 CO-NDM donor strain, and Dr. Mark Fisher for providing the clinical *A. baumannii* recipient
460 strain.
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